Biochemical Pharmacology, Vol. 42, No. 10, pp. 2003–2009, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

DIFFERENTIAL INHIBITION OF EPIDERMAL GROWTH FACTOR BINDING BY PHOTOACTIVATED PSORALENS AND THE TUMOR PROMOTER 12-*O*-TETRADECANOYL PHORBOL-13-ACETATE IN CELLS OVEREXPRESSING PROTEIN KINASE C

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(*Received* 26 *February* 1991; *accepted* 21 *June* 1991)

Abstract—A rat fibroblast cell line, R6PKC3, that stably overexpresses the β-1 form of protein kinase C was used to analyze sensitivity to inhibitors of epidermal growth factor (EGF) binding. R6PKC3 cells overexpress protein kinase C activity 53-fold relative to non-overexpressing control R6C1 cells. Inhibition of EGF binding by the tumor promoter 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) and photoactivated psoralens was compared in these cells. We found that ^{125}I-EGF bound both of the cell lines and was rapidly internalized in a temperature-dependent process and metabolized. Binding of EGF to the R6 cells overexpressing protein kinase C was markedly less than binding to R6C1 control cells. In both of the cell lines, TPA and photoactivated psoralens inhibited ^{125}I-EGF binding but the response of these cells to these inhibitors was distinct. R6PKC3 cells were markedly more sensitive to TPA and were resistant to recovery from TPA-induced inhibition of ^{125}I-EGF binding when compared to control cells. These differences were not observed in other subclones of cells overexpressing protein kinase C, suggesting that they may be unique to R6PKC3 cells. In contrast, no major differences in sensitivity to photoactivated psoralens were observed in R6C1 and R6PKC3 cells. These data indicate that TPA and photoactivated psoralens inhibit ^{125}I-EGF binding to these cell lines by distinct mechanisms.

A number of studies have implicated the calcium- and phospholipid-dependent enzyme, protein kinase C, as an important mediator of cellular transformation and growth control [1–3]. The enzyme is widely distributed in a variety of tissues and is known to exist in a number of discrete forms which vary in tissue distribution [4]. The expression of different isoforms of protein kinase C may be important in either modifying or selectively mediating responses to different stimuli in various cell types.

One protein known to be a substrate for protein kinase C is the receptor for epidermal growth factor (EGF) [5–7]. The EGF receptor is a 170,000 molecular weight transmembrane glycoprotein possessing intrinsic tyrosine-specific protein kinase activity that is activated by EGF [8–13]. The receptor protein is phosphorylated on threonine and serine residues and, following EGF binding, at tyrosine residues through a process of autophosphorylation [14]. One of the most well characterized inhibitors of EGF binding is the tumor promoter, 12-*O*-tetradecanoyl phorbol-13-acetate (TPA). By directly binding to and activating protein kinase C, TPA induces phosphorylation of the EGF receptor [6]. This presumably alters the conformation of the EGF receptor, preventing ligand binding [15, 16]. Our laboratory has also shown that the photoactivated psoralens are potent inhibitors of EGF binding [17]. Like TPA, photoactivated psoralens stimulate EGF receptor phosphorylation [18]. Photoactivated psoralens are potent modulators of epidermal cell growth and differentiation, although their mechanism of action appears to be distinct from TPA [17, 18]. Both TPA and the photoactivated psoralens also reduce EGF-stimulated EGF receptor tyrosine kinase activity [6, 18, 19].

In the present studies we characterized EGF binding and inhibition of binding by TPA and photoactivated psoralens in two rat fibroblast-derived cell lines, R6C1 and R6PKC3. R6PKC3 cells, which have been transfected with the β-1 form of protein kinase C, have been reported to express 53-fold greater activity of protein kinase C than control R6 cells [20, 21]. We found that EGF readily bound to cell membrane receptors and was subsequently internalized and metabolized in both cell types. However, less EGF binding was observed in R6PKC3 cells when compared to R6C1 control cells. In addition, although there were no differences in the sensitivity of EGF receptors to inhibition by the photoactivated psoralens, significantly lower concentrations of TPA were required to inhibit EGF binding in overexpressing R6 cells. These results indicate that, in these cells, the actions of TPA and the photoactivated psoralens are distinct.

MATERIALS AND METHODS

Reagents. 4,5′,8-Trimethylpsoralen (TMP) was obtained from Elder Pharmaceuticals (Bryant, OH). TPA and chloroquine were purchased from Sigma

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(St. Louis, MO). EGF was isolated from the submaxillary glands of mice and purified as described by Savage and Cohen [22]. ^{125}I-Labeled EGF (sp. act. 180 μCi/μg) was from New England Nuclear (Wilmington, DE).

Cell culture. The cell lines R6C1 and R6PKC3 were derived from R6 rat fibroblasts and were provided by Dr. I. Bernard Weinstein, Columbia University. Cells overexpressing protein kinase C (R6PKC3) were constructed using a retroviral expression vector containing the full length cDNA for the β-1 isoform of protein kinase C [20]. The control cell line (R6C1) carries an integrated expression vector lacking the cDNA insert. The cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% calf serum, 100 units/mL penicillin, 100 μg/mL streptomycin and 50 μg/mL of the Neomycin derivative G-418 (Gibco, Grand Island, NY) in a humidified incubator at 37° and 5% CO_2.

Binding assays. For binding assays, cells (1×10^5) were inoculated into 60 mm tissue culture dishes and incubated for 2–3 days. Cells were then washed with phosphate-buffered saline (PBS) and treated with either TPA or TMP as indicated below at 37° for 30 min in 2 mL of binding buffer consisting of DMEM containing 50 mM HEPES buffer, pH 7.5, and 1 mg/mL of bovine serum albumin. Cells were then washed with PBS, and 1.5 to 2 mL of binding buffer supplemented with 22 pM ^{125}I-EGF (180 μCi/μg) was added to each plate. Non-specific binding was determined by incubating the cells with binding buffer containing the radioligand and excess unlabeled EGF (1 μg/mL). Cells were incubated for 1.5 to 2 hr at 37° and then washed with ice-cold PBS (5×2 mL). Two milliliters of 0.2 N NaOH were used to solubilize the cells, and radioactivity was determined using a gamma counter. Scatchard analysis of ^{125}I-EGF binding was performed as previously described [17].

Treatment of cells with PUVA and TPA. Cells were treated with TPA or the combination of TMP and ultraviolet light (UVA), a procedure referred to as PUVA, as previously described [17]. Briefly, TMP or TPA was added directly to the binding buffer. For PUVA experiments, cells, treated with TMP, were irradiated with UVA light emitted from a bank of four BLB fluorescent light bulbs (F40 BL, Sylvania) placed 10 cm above the cell culture plates. Incident light on the culture dishes was 2.8 mW/cm^2 as determined with an International Light ultraviolet radiometer (model 442) fitted with an IL-SE 115 probe and a 363 UVA pass filter.

Recovery of ^{125}I-EGF binding following TPA treatment. To examine the recovery of EGF receptors following TPA treatment, cells were rinsed with 5 mL of binding buffer and incubated for 20 min at 37° with 2 mL of binding buffer supplemented with 32 nM TPA. Cells were then rinsed twice with 2 mL of binding buffer and refed with 4 mL of DMEM containing 10% calf serum. After increasing periods of time up to 24 hr, ^{125}I-EGF binding was measured as described above.

Measurement of internalization and metabolism of ^{125}I-EGF. Internalization of ^{125}I-EGF by the cells was quantified using the method of Haigler *et al.* [23]. Briefly, cells were washed once with PBS and then incubated with 2 mL of binding buffer containing ^{125}I-EGF (22 pM). After appropriate time intervals the cells were washed with ice-cold PBS (5×2 mL) to remove unbound label. One milliliter of ice-cold acidic stripping buffer (0.2 N acetic acid, pH 2.5, 0.5 M NaCl) was then added to each dish. Six minutes later, the stripping buffer was removed and the cells were washed with an additional 0.5 mL of this buffer. Washes were collected for determination of released radioactivity. Adherent cells were solubilized in 2 mL of 0.2 N NaOH and counted for radioactivity. In these assays, ^{125}I-EGF binding was determined both at 37° and at 4°, which has been shown to inhibit EGF internalization [24].

The metabolism of EGF was analyzed according to the method of Carpenter and Cohen [24]. Briefly, the cells were seeded into 24-well culture plates and grown for 2–3 days. The cells were then incubated with 0.5 mL of binding buffer, with or without chloroquine (100 μM) for 20 min at 37°. Binding buffer (0.5 mL) containing 44 pM ^{125}I-EGF was added directly to each well and allowed to equilibrate for 2 hr at 4°. Cells were then incubated at 37° for 20 min to permit internalization of bound radiolabel and washed to remove residual radioactivity before the addition of 0.5 mL of fresh label-free binding buffer. In some experiments, chloroquine was also added to the labeling medium and binding buffer. Aliquots (400 μL) of the binding buffer were removed at the indicated times and counted to determine total release of radioactivity. ^{125}I-EGF in the medium was determined following the addition of ice-cold trichloroacetic acid (TCA, 10% final concentration) and placing the samples on ice for 1 hr. Precipitates were collected following centrifugation, washed twice with 10% TCA, and counted for radioactivity.

RESULTS

Characterization of ^{125}I-EGF binding in cells overexpressing protein kinase C. In initial studies we compared the binding of ^{125}I-EGF to control R6C1 cells with binding to R6PKC3 cells overexpressing protein kinase C. We found that ^{125}I-EGF readily bound to each of the cell types. As shown in Table 1, specific binding represented over 92% of total binding of EGF to the cells. For each of the cell lines, specific binding of ^{125}I-EGF was found to be temperature dependent. Greater ^{125}I-EGF binding was observed at 37° when compared to 4°. We found that ^{125}I-EGF binding to R6 cells overexpressing protein kinase C was consistently lower than binding to control R6 cells at both 4° and 37°. A Scatchard analysis performed at 4° revealed that the K_d for ^{125}I-EGF binding in both of the R6 cells lines was generally similar (Table 2). However, we did observe a decrease in B_{max} in the R6PKC3 cells and this may account, at least in part, for the lower amount of ^{125}I-EGF binding to these cells when compared to the non-overexpressing cells.

Internalization and degradation of ^{125}I-EGF. Once bound to cells, ^{125}I-EGF is internalized in a temperature-dependent process [24]. We found that at 37°, 80–90% of the ^{125}I-EGF bound to both the R6 cell lines was internalized within 60 min (Fig. 1).

Table 1. ^{125}I-EGF binding to R6 cells

	^{125}I-EGF binding* (cpm/10^6 cells)	
	R6C1	R6PKC3
Total binding		
No treatment	4270 ± 70	3510 ± 110
+Unlabeled EGF (1 μg/mL)	270 ± 10	280 ± 20
Specific binding		
No treatment	4010 ± 50	3230 ± 80
+UVA (2.8 J/cm^2)	2330 ± 100	2020 ± 70
+TPA (17 nM)	2590 ± 20	410 ± 60
+PUVA†	1310 ± 110	1260 ± 60

* Cells were assayed for ^{125}I-EGF binding at 37° for 1.5 hr. Each value is the average ± range of duplicate samples.

† Cells were treated with 4.4 μM TMP followed by 2.8 J/cm^2 UVA light as described in Materials and Methods.

Table 2. Scatchard analysis of ^{125}I-EGF binding to R6 cells

Cell lines	K_d (pM)	B_{max} (pmol/10^6 cells)
R6C1	2.6	0.038
R6PKC3	1.5	0.026

^{125}I-EGF specific binding for Scatchard analysis was performed with increasing concentrations of the radioligand as indicated in Materials and Methods.

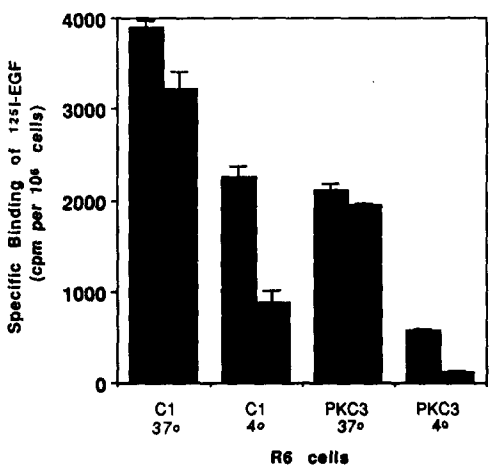

Fig. 1. Characterization of cell surface-associated and internalized ^{125}I-EGF. Cells were washed with PBS and then incubated with 22 pM ^{125}I-EGF at 4° for 2.5 hr or at 37° for 1 hr. Surface bound ^{125}I-EGF was removed with an acetic acid stripping buffer. To quantify the remaining cell-associated ^{125}I-EGF, the cells were solubilized in 0.2 N NaOH and counted for radioactivity as described in Materials and Methods. Key: solid bars, total cell-associated ^{125}I-EGF; shaded bars, internalized ^{125}I-EGF. Each value is the average of triplicate samples ± SD.

A marked decline in ^{125}I-EGF internalization was observed in each cell type at 4° (Fig. 1).

Following internalization, ^{125}I-EGF is rapidly metabolized in lysosomes and the degradation products are released into the cell culture medium [24]. Intact ^{125}I-EGF can be precipitated from the medium with TCA while small degradation products remain soluble. In metabolism experiments, we found that ^{125}I-EGF and its degradation products could be identified in the culture medium of R6C1 and R6PKC3 cells (Fig. 2). In R6 control cells, approximately 57% of the radioactivity released was in the form of metabolites of ^{125}I-EGF, while in R6 cells overexpressing protein kinase C, 85% was metabolized. After 6 hr, the overexpressing R6 cells were also found to release more of the total cell-associated radioactivity into the medium when compared to the control cells.

In both R6 cell types we found that chloroquine, an inhibitor of lysosomal degradation of EGF, suppressed ^{125}I-EGF metabolism as indicated by the appearance of increased amounts of acid-precipitable material in the cell culture medium (Fig. 2). Intact EGF accounted for 55–58% of the label released into the medium within 6 hr following chloroquine treatment. In cells overexpressing protein kinase C, the total radioactive material released into the medium decreased following chloroquine treatment. In control cells, total radioactivity released into the medium also decreased following chloroquine treatment but to a much smaller extent when compared to the R6PKC3 cells. Chloroquine alone had no effect on the binding of ^{125}I-EGF to any of the cells (data not shown).

Effects of TPA and PUVA on ^{125}I-EGF binding. Previous studies have demonstrated that treatment of cells with either TPA [5, 6] or PUVA [18] results in a marked reduction of ^{125}I-EGF receptor binding. Similarly, in the present studies we found that both of these treatments were effective in inhibiting ^{125}I-EGF binding to cells overexpressing protein kinase C as well as control cells (Table 1 and Figs. 3 and 4). Overexpressing R6 cells were found to be markedly more sensitive to the inhibitory effects of TPA than were the R6 control cells. The concentration of TPA inhibiting ^{125}I-EGF binding by 50% (IC_{50}) in each cell type was 0.9 and 33 nM, respectively.

In further experiments, we found that while TMP

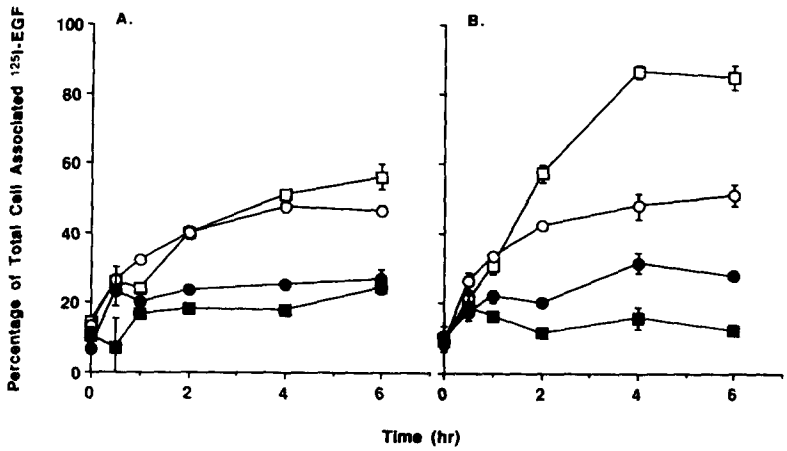

Fig. 2. Metabolism of ^{125}I-EGF. Cells were incubated with ^{125}I-EGF for 2 hr at 4°. Following internalization at 37° for 20 min, cells were washed to remove excess label and label-free binding buffer was added to the plates. Aliquots of the medium were removed at the indicated times for determination of intact and metabolized ^{125}I-EGF. Total cell-associated radioactivity was determined as the specific cell-bound ^{125}I-EGF following binding. (A) R6C1 cells; (B) R6PKC3 cells. Total radioactivity released into the medium in the absence (□) or presence (○) of chloroquine is represented as the fraction of total cell-associated ^{125}I-EGF. Also shown is acid-precipitable radioactivity released into the medium in the absence (■) and presence (●) of chloroquine. Each value is the average of triplicate samples ± SD.

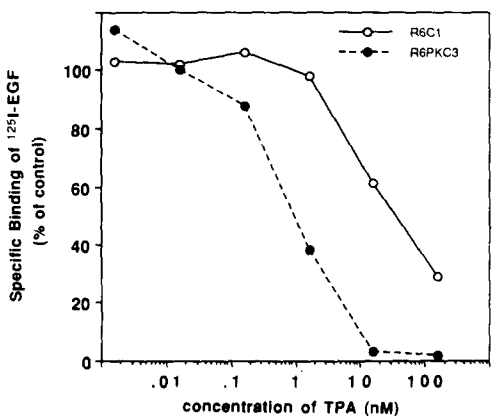

Fig. 3. Concentration-dependent inhibition of ^{125}I-EGF binding by TPA. Cultures were pretreated with increasing concentrations of TPA in 2 mL of binding buffer for 30 min at 37° and then assayed for ^{125}I-EGF binding as described in Materials and Methods. Non-specific binding was determined by the addition of an excess of unlabeled EGF. Specifically bound ^{125}I-EGF is expressed as a percentage of untreated controls. Specific binding in the absence of TPA was 1780 cpm/dish for R6C1 cells and 1300 cpm/dish for R6PKC3 cells. Each value is the average of duplicate samples.

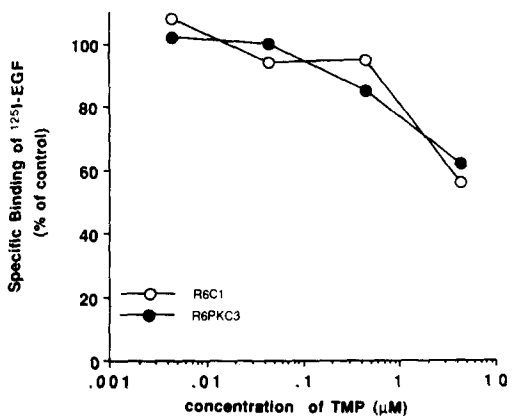

Fig. 4. Concentration-dependent inhibition of ^{125}I-EGF binding by photoactivated psoralens. Cells were pretreated with increasing concentrations of TMP in 2 mL of binding buffer for 30 min at 37° and then irradiated with 2.8 J/cm^2 of UVA light. ^{125}I-EGF binding was assayed as described in Materials and Methods. Specific ^{125}I-EGF binding is expressed as a percentage of untreated controls. Specific binding in the absence of PUVA treatment was 4410 cpm/10^6 cells for R6C1 cells and 3810 cpm/10^6 cells for R6PKC3 cells. Each value is the average of duplicate samples.

alone had no effect on ^{125}I-EGF binding to the R6 cells (data not shown), UVA light partially inhibited binding although not as effectively as the combination of TMP and UVA light (Table 1). In contrast to TPA, no differences were observed in sensitivity of R6 cells to PUVA-induced inhibition of ^{125}I-EGF binding (Fig. 4).

In separate experiments we determined if inhibition of EGF binding by PUVA was associated with alterations in EGF metabolism. Figure 5 shows that both PUVA and chloroquine inhibited the release of labeled EGF and its metabolites from R6C1 cells. This was associated with a small decrease in metabolism of ^{125}I-EGF by these cells. R6PKC3 cells metabolized a greater percentage of total cell-associated ^{125}I-EGF when compared to R6C1 cells. In R6PKC3 cells, PUVA and chloroquine also

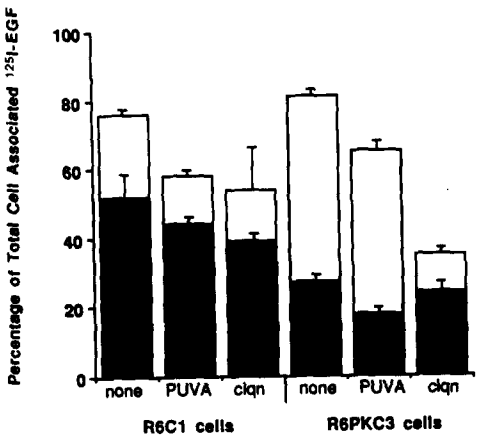

Fig. 5. Effects of PUVA and chloroquine on ^{125}I-EGF metabolism in R6 cells. ^{125}I-EGF metabolism was analyzed in each of the cell lines following either photoactivated psoralen or chloroquine treatment as described in Materials and Methods. Unmetabolized ^{125}I-EGF released from the cells into the culture medium was quantified following TCA precipitation. Key: open bars, metabolized plus unmetabolized ^{125}I-EGF released from the cells; solid bars, unmetabolized ^{125}I-EGF released from the cells. Each value is the average of triplicate samples ± SD.

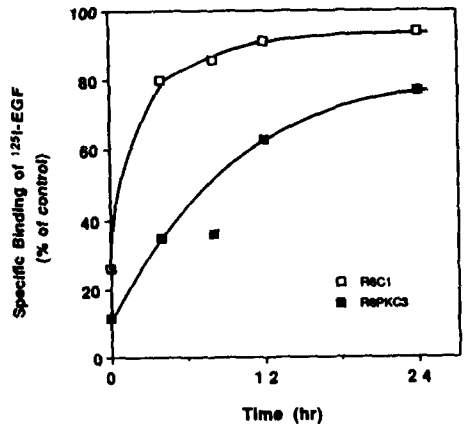

Fig. 6. Recovery of ^{125}I-EGF binding following TPA treatment. Cells were treated with TPA, washed and incubated for the indicated time periods. ^{125}I-EGF binding was then assayed as described in Materials and Methods. Non-specific binding was determined by quantifying binding in the presence of excess unlabeled EGF. Each value is the average of duplicate samples. Specific binding in R6C1 and R6PKC3 cells for 0-hr controls was 3190 and 4770 cpm/10^6 cells, respectively.

reduced the release of labeled EGF and its metabolites from the cells. In addition, chloroquine was markedly more effective in inhibiting ^{125}I-EGF metabolism in these cells when compared to R6C1 cells.

Recovery of ^{125}I-EGF binding after TPA treatment. Inhibition of ^{125}I-EGF binding by TPA, but not PUVA, is known to be reversible [18, 25]. In control R6 cells, we found that recovery of EGF receptor binding following TPA treatment was rapid, largely occurring within 4 hr (Fig. 6). Recovery of EGF receptor binding in R6 cells overexpressing protein kinase C was found to be delayed 4–8 hr when compared to control R6 cells. In separate studies we tested additional cell lines that overexpress protein kinase C [26] and found no differences in their sensitivity to TPA or to photoactivated psoralens (data not shown). In these studies, similar concentrations of TPA were found to inhibit ^{125}I-EGF binding in both cells overexpressing protein kinase C and their non-overexpressing counterparts. Taken together, these data suggest that differences in sensitivity of cells to TPA may be unique to the R6PKC3 cell line.

Morphological alterations in R6 cells following treatment with TPA and PUVA. We also noted that the effects of TPA and PUVA on the morphology of the R6 cells were distinct. As previously described by Housey *et al.* [20], in the absence of TPA or PUVA, confluent monolayer cultures of R6PKC3 and R6C1 cells display a characteristic fusiform morphology. Within 6 hr after treatment with 100 ng/mL of TPA, R6PKC3 cells became refractile, rounded on the culture dishes and displayed prominent elongated cytoplasmic processes ([20] and data not shown). In contrast, no morphological changes were apparent in R6C1 cells treated with TPA for 6 hr ([20] and data not shown) or in either R6C1 or R6PKC3 cells after treatment with PUVA (not shown). Taken together, these data indicate that the actions of TPA and PUVA on R6 cells are distinct and that the morphological changes induced by TPA may not necessarily be related to its inhibitory effects on EGF binding.

DISCUSSION

In this paper we examined EGF receptor binding and inhibition of binding by photoactivated psoralens and TPA in a rat fibroblast cell line overexpressing the β-1 form of protein kinase C and its non-overexpressing counterpart. We found that both cell types readily bind ^{125}I-EGF, and internalize and metabolize the ligand. In cells overexpressing protein kinase C, less ^{125}I-EGF was bound at 4°, when compared to non-overexpressing cells. This was apparently due to fewer EGF receptors on these cells. Using a retroviral expression system, Eldar *et al.* [27] recently produced cell lines from Swiss 3T3 mouse fibroblasts that overexpress the α subtype of protein kinase C. Like R6 cells overexpressing protein kinase C, the 3T3 cells contained fewer EGF receptors. Taken together, these data indicate that changes in expression of EGF receptors in cells overexpressing protein kinase C may be independent of the isoform of protein kinase C overexpressed in the cells as well as the species-origin of the cells.

Following receptor binding, EGF is internalized in a temperature-dependent process by receptor-mediated endocytosis [24]. In the present paper, ^{125}I-EGF binding and internalization were also temperature dependent in both R6C1 and R6PKC3 cells; no significant differences in internalization of

the radioligand were observed between the cell lines. In addition, both cell lines effectively metabolized ^{125}I-EGF in a process that was inhibitable by chloroquine. A greater percentage of EGF taken up by R6PKC3 cells was metabolized when compared to R6C1 cells. This suggests that changes in levels of protein kinase C alter the metabolism of EGF. Interestingly, chloroquine was found to be much more effective in inhibiting the metabolism of ^{125}I-EGF in the cells overexpressing protein kinase C when compared to the control cells. These data suggest that chloroquine sensitivity may be influenced by intracellular levels of protein kinase C.

Both photoactivated psoralens and TPA were found to inhibit ^{125}I-EGF binding to the different cell lines. The photoactivated psoralens were not as effective as TPA in inhibiting ^{125}I-EGF receptor binding in these cells and this may be due to the distinct mechanisms of action of these compounds (see further below). Interestingly, R6PKC3 cells were markedly more sensitive to the effects of TPA than were control R6C1 cells. The increased sensitivity of R6PKC3 cells to TPA may be due to the significantly greater amounts of protein kinase C in these cells. It is also possible that other biochemical factors such as differences in the subcellular distribution of protein kinase C after TPA treatment [28, 29], the degree of protein kinase C-induced phosphorylation of the EGF receptor [6, 19, 30], and/or the presence of endogenous activators of the enzyme such as diacylglycerol [31] contribute to the differential sensitivity to TPA. Recently, Pai *et al.* [21] reported that TPA induces diacylglycerol production in both R6C1 and R6PKC3 cells. TPA-inducible diacylglycerol, which was thought to result from enhanced phospholipase D activity, was found to be much greater in R6PKC3 cells than in R6C1 cells, supporting the model that this lipid may play a role in the increased sensitivity of R6PKC3 cells to TPA-induced inhibition of EGF binding. R6 cells overexpressing protein kinase C also possess fewer EGF receptors and this may be important in the decreased levels of TPA required to inhibit ^{125}I-EGF binding. Alternatively, it may simply be a property of the clonal isolates used for this work.

It was of interest that R6PKC3 cells were not only more sensitive to EGF binding inhibition by TPA when compared to control R6C1 cells, but they were also more resistant to recovery from TPA-induced inhibition of EGF binding. Previous studies have shown that inhibition of EGF binding by TPA is readily reversible [17, 25]. The slower recovery of the R6PKC3 cells following TPA treatment may also be related to levels of protein kinase C in the cells. This may be important if larger amounts of the activated enzyme remain associated with the cell membranes following the reappearance of EGF receptors on the cell surface. Eldar *et al.* [27] provided evidence that levels of the α form of protein kinase C could also regulate expression of EGF receptor mRNA and protein. Further studies are required to determine the extent to which levels of the β-1 form of protein kinase C are important in regulating expression of EGF receptors in the R6 cell lines. As indicated above, chloroquine inhibited EGF metabolism in each of the R6 cell lines. Although PUVA was effective in inhibiting EGF binding in R6 cells, only a relatively small inhibition of EGF metabolism was observed. These data support the idea that the biological effects of the psoralens are limited largely to the cell surface membrane [32]. In this regard, our laboratory has shown that the psoralens bind to high-affinity receptor sites located in membrane fractions of responsive cell types [33, 34].

In summary, the present studies address the question of whether levels of protein kinase C in R6PKC3 cells play a role in determining sensitivity to two inhibitors of EGF binding, TPA and photoactivated psoralens. For TPA, a direct activator of protein kinase C, sensitivity to inhibition may be related to levels of protein kinase C. In contrast, sensitivity of cells to photoactivated psoralens appears to be largely independent of cellular levels of protein kinase C. We have shown previously that the psoralen receptor is distinct from protein kinase C both in terms of molecular size and ligand binding specificity [33, 34]. In addition, psoralens do not compete directly with the phorbol esters for binding to protein kinase C [17, 33] and, unlike TPA, are unable to activate the enzyme in purified preparations. Psoralens also induce unique patterns of phosphorylation of membrane proteins in cells, including the EGF receptor, when compared to TPA [18]. Earlier studies have demonstrated alterations in the morphology of the R6 cell lines following prolonged treatment with TPA [20]. The present work shows that in R6PKC3 cells, these alterations also occur within several hours. The fact that these changes in cell structure are not found in cells treated with the photoactivated psoralens also indicates that these compounds are not activating protein kinase C. Taken together, our data support the model that there are distinct mechanisms of action for TPA and the psoralens in responsive cell types.

Acknowledgement—This work was supported by NIEHS Grant ES 03647 awarded to J. D. Laskin.

REFERENCES

1. Nishizuka Y, The role of protein kinase C in cell surface signal transduction and tumour promotion. *Nature* **308**: 693–698, 1984.
2. Nishizuka Y, Studies and perspectives of protein kinase C. *Science* **233**: 305–312, 1986.
3. Blumberg PM, Protein kinase C as the receptor for the phorbol ester tumor promoters. *Cancer Res* **48**: 1–8, 1988.
4. Nishizuka Y, The molecular heterogeneity of protein kinase C and its implications for cellular regulation. *Nature* **334**: 661–665, 1988.
5. Gill GN and Lazar CS, Increased phosphotyrosine content and inhibition of proliferation in EGF-treated A431 cells. *Nature* **293**: 305–307, 1981.
6. Cochet C, Gill GN, Meisenhelder J, Cooper JA and Hunter T, C-kinase phosphorylates the epidermal growth factor receptor and reduces its epidermal growth factor-stimulated tyrosine protein kinase activity. *J Biol Chem* **259**: 2553–2558, 1984.
7. Iwashita S and Fox CF, Epidermal growth factor and potent phorbol tumor promoters induce epidermal growth factor receptor phosphorylation in a similar but

distinctly different manner in human epidermal carcinoma A431 cells. *J Biol Chem* **259**: 2559–2567, 1984.
8. Nexo E, Hock RA and Hollenberg MD, Lectin-agarose immobilization, a new method for detecting soluble membrane receptors: Applications to studies with epidermal growth factor-urogastrone and transcobalamin-II. *J Biol Chem* **254**: 8740–8743, 1979.
9. Cohen S, Ushiro H, Stoscheck C and Chinkers M, A native 170,000 epidermal growth factor receptor-kinase complex from shed plasma membrane vesicles. *J Biol Chem* **257**: 1523–1531, 1982.
10. Buhrow SA, Cohen S, Garbers DL and Staros JV, Characterization of the interaction of 5′-*p*-fluorosulfonylbenzoyl adenosine with the epidermal growth factor receptor protein kinase in A431 cell membranes. *J Biol Chem* **258**: 7824–7827, 1983.
11. Downward J, Parker P and Waterfield MD, Autophosphorylation sites on the epidermal growth factor receptor. *Nature* **311**: 483–485, 1984.
12. Weber W, Bertics PJ and Gill GN, Immunoaffinity purification of the epidermal growth factor receptor. *J Biol Chem* **259**: 14631–14636, 1984.
13. Carpenter G, Receptors for epidermal growth factor and other polypeptide mitogens. *Annu Rev Biochem* **56**: 881–994, 1987.
14. Hunter T and Cooper JA, Protein tyrosine kinases. *Annu Rev Biochem* **54**: 897–930, 1985.
15. Lee LH and Weinstein IB, Tumor-promoting phorbol esters inhibit binding of epidermal growth factor to cellular receptors. *Science* **202**: 313–315, 1978.
16. Shoyab M, Delarco JE and Todaro GJ, Biologically active phorbol esters specifically alter affinity of epidermal growth factor membrane receptors. *Nature* **279**: 387–391, 1979.
17. Laskin JD, Lee E, Laskin DL and Gallo MA, Psoralens potentiate ultraviolet light-induced inhibition of epidermal growth factor binding. *Proc Natl Acad Sci USA* **83**: 8211–8215, 1986.
18. Mermelstein FH, Abidi TF and Laskin JD, Inhibition of epidermal growth factor receptor tyrosine kinase activity in A431 human epidermoid cells following psoralen/ultraviolet light treatment. *Mol Pharmacol* **36**: 848–855, 1989.
19. Friedman B, Frackelton AR, Ross AH, Connors JM, Fujiki H, Sugimura T and Rosner MR, Tumor promoters block tyrosine-specific phosphorylation of the epidermal growth factor receptor. *Proc Natl Acad Sci USA* **81**: 3034–3038, 1984.
20. Housey GM, Johnson MD, Hsiao WLW, O'Brian CA, Murphy JP, Kirschmeier P and Weinstein IB, Overproduction of protein kinase C causes disordered growth control in rat fibroblasts. *Cell* **52**: 343–354, 1988.
21. Pai J-K, Pachter JA, Weinstein IB and Bishop WR, Overexpression of protein kinase C β1 enhances phospholipase D activity and diacylglycerol formation in phorbol ester-stimulated rat fibroblasts. *Proc Natl Acad Sci USA* **88**: 598–602, 1991.
22. Savage CR and Cohen S, Epidermal growth factor and a new derivative. *J Biol Chem* **247**: 7609–7611, 1972.
23. Haigler HT, Maxfield FR, Willingham MC and Pastan I, Dansylcadaverine inhibits internalization of ^{125}I-epidermal growth factor in BALB 3T3 cells. *J Biol Chem* **255**: 1239–1241, 1980.
24. Carpenter G and Cohen S, ^{125}I-Labelled human epidermal growth factor (hEGF): Binding, internalization and degradation in human fibroblasts. *J Cell Biol* **71**: 159–171, 1976.
25. Lee LS and Weinstein IB, Mechanism of tumor promoter inhibition of cellular binding of epidermal growth factor. *Proc Natl Acad Sci USA* **76**: 5168–5172, 1979.
26. Krauss RS, Housey GM, Johnson MD and Weinstein IB, Disturbances in growth control and gene expression in a C3H/10T1/2 cell line that stably overproduces protein kinase C. *Oncogene* **4**: 991–998, 1989.
27. Eldar H, Zisman Y, Ullrich A and Liveneh E, Overexpression of protein kinase C α-subtype in Swiss/3T3 fibroblasts causes loss of both high and low affinity receptor numbers for epidermal growth factor. *J Biol Chem* **265**: 13290–13296, 1990.
28. Kraft AS, Anderson WB, Cooper HL and Sando JJ, Decrease in cytosolic calcium/phospholipid-dependent protein kinase activity following phorbol ester treatment of EL4 thymoma cells. *J Biol Chem* **257**: 13193–13196, 1982.
29. Kraft AS and Anderson WB, Phorbol esters increase the amount of Ca^{2+}, phospholipid-dependent protein kinase associated with plasma membrane. *Nature* **301**: 621–623, 1983.
30. McCaffrey PG, Friedman B and Rosner MR, Diacylglycerol modulates binding and phosphorylation of the epidermal growth factor receptor. *J Biol Chem* **259**: 12502–12507, 1984.
31. Castagna M, Takai Y, Kaibuchi K, Sano K, Kikkawa U and Nishizuka Y, Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. *J Biol Chem* **257**: 7847–7851, 1982.
32. Laskin JD and Laskin DL, Role of psoralen receptors in cell growth regulation. In: *Psoralen DNA Photobiology* (Ed. Gasparro FP), Vol. II, pp. 135–148. CRC Press, Boca Raton, FL, 1988.
33. Laskin JD, Lee E, Yurkow EJ, Laskin DL and Gallo MA, A possible mechanism for psoralen phototoxicity not involving direct interaction with DNA. *Proc Natl Acad Sci USA* **82**: 6158–6162, 1985.
34. Yurkow EJ and Laskin JD, Characterization of a photoalkylated psoralen receptor in HeLa cells. *J Biol Chem* **262**: 8439–8442, 1987.